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Ivermectin-facilitated immunity in onchocerciasis. Reversal of lymphocytopenia, cellular anergy and deficient cytokine production after single treatment

P. T. SOBOSLAY, C. M. DREWECK, W. H. HOFFMANN, C. G. K. LÜDER, C. HEUSCHKEL, H. GÖRGEN*, M. BANLA† & H. SCHULZ-KEY *Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany, †Centre Hospitalier du Région, Sokodé, Togo, and *Deutsche Gesellschaft für Technische Zusammenarbeit (gtz), Eschborn, Germany*

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SUMMARY

A longitudinal investigation has been conducted into the cell-mediated immune responses of onchocerciasis patients after a single-dose treatment with ivermectin. Untreated patients tested for delayed cutaneous hypersensitivity (DCH) to seven recall antigens showed lower responses than infection-free control individuals ($P < 0{\cdot}01$), but 6 and 14 months after treatment DCH reactions increased to similar levels to those seen in the controls. The *in vitro* cellular reactivity to *Onchocerca volvulus*-derived antigen (OvAg) was reduced in untreated patients as compared with controls, and the lymphocyte blastogenic responses to OvAg and streptolysin-O clearly improved up to 14 months after treatment. Peripheral blood mononuclear cells (PBMC) from untreated patients produced IL-1β, tumour necrosis factor-alpha (TNF-α) and IL-6 in response to mitogenic stimulation with phytohaemagglutinin (PHA), only low levels of IL-1β, IL-2 and TNF-α in response to OvAg, but higher amounts of IL-4 and interferon-gamma (IFN-γ) in response to OvAg than control individuals. After ivermectin treatment, the OvAg-induced production of IL-1β and TNF-α increased significantly 1 and 14 months after treatment. The PHA-induced production of IL-2 and IL-4 increased 1 month after treatment and remained significantly elevated until 14 months after treatment, whereas the OvAg-specific secretion of IL-2, IL-4 and IFN-γ did not change after ivermectin treatment. Flow cytometric analysis of lymphocyte-subsets in the peripheral blood of untreated patients revealed a relative and absolute ($P < 0{\cdot}01$) diminution of $CD4^+$ cells and a significantly smaller $CD4^+/CD8^+$ cell ratio as compared with controls. By 4 weeks after treatment and thereafter, $CD4^+$ T cells increased relatively and absolutely ($P < 0{\cdot}01$); likewise there was an absolute increase in T-helper-inducer cells ($CD4^+CD45RO^+$) and a temporarily improved $CD4^+/CD8^+$ cell ratio ($P = 0{\cdot}001$). The expression of the low-affinity receptor for IgE (CD23) on total lymphocytes decreased from 14% to 7% by 14 months after treatment. The $CD8^+$ cells and $CD3^+TCR\gamma\delta^+$ cells were higher in patients than in controls and both remained elevated until 14 months after treatment. These results suggest a distinctly improved cellular immunity in human onchocerciasis that was facilitated by ivermectin therapy.

Keywords onchocerciasis ivermectin cellular immunity cytokines lymphocyte subpopulations

INTRODUCTION

In humans chronically infected with *Wuchereria bancrofti* [1] or *Onchocerca volvulus* [2,3] cellular unresponsiveness to parasite antigen is associated with the inability to produce IL-1, IL-2 and interferon-gamma (IFN-γ). The loss of T cell reactivity against filarial antigens has been observed to coincide with the onset of microfilaraemia [4–6]. These deficiencies are regarded as multifactorial and as independent defects of the T cell activation pathway [7]. Cellular unresponsiveness to filarial and bacterial antigens was partially reversed *in vitro* after addition of exogenous IL-2 [3], and *in vivo* after treatment of patients with diethylcarbamazine (DEC) [8,9], but severe side effects of the kind induced by DEC make this drug unsuitable for community-based mass treatment. On the other hand, onchocerciasis is effectively treated with ivermectin [10], a microfilaricide with

Correspondence: Dr P. T. Soboslay, Institute of Tropical Medicine, University of Tübingen, Wilhelmstr. 27, 7400 Tübingen, Germany.

moderate side effects [11] and a long-lasting efficacy which is regarded as a major contribution to the suppression of disease development [12]. The short half-life of ivermectin in human plasma [13] and the transient effect on the adult worm [14] stand in contrast to a persistent reduction of microfilaridermia. Recently, Steel *et al.* [15] found cellular responses of onchocerciasis patients to be transiently activated after repeated doses of ivermectin. Also, the functional abnormalities and phenotypic distribution of lymphocyte subsets in patients changed during therapy [7]. However, defects in the T cell activation pathway remained, even though a relative reversal was registered. Since ivermectin is now being used for mass treatment of onchocerciasis, detailed knowledge of the immunological mechanisms facilitated by ivermectin are of importance. A restitution of immunity might support the elimination of microfilariae or prevent new infection in infected individuals. This study reports on cellular reactivity, cytokine production and phenotypic distribution of peripheral blood lymphocyte subsets in onchocerciasis patients following a single ivermectin therapy regime.

PATIENTS AND METHODS

Study population

Sixty-one onchocerciasis patients from a hyperendemic area in central Togo volunteered to participate in our study. All patients received a thorough parasitological and clinical examination which included physical examination, total leucocyte counts and blood cell differential, assessment of microfilarial densities in skin snips, palpation for onchocerca nodules, diagnosis of concomitant parasitic infections in stool and blood specimens, and a slit lamp examination for the presence of intraocular microfilariae of *O. volvulus*. Patients were treated with a single dose of 150 μg/kg ivermectin. Along with clinical and parasitological examinations, blood specimens were taken before therapy, at 1 week, 1 month, 6 months and 14 months after treatment. Eight residents from Lomé and Sokodé (Togo) found to be negative for microfilariae of *O. volvulus* volunteered as local controls. All participants included in this study were HIV-I and HIV-II antibody-negative as determined by IgG ELISA.

Isolation of PBMC

Heparinized blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Pharmacia) density gradient centrifugation. PBMC were adjusted to 1×10^7/ml in RPMI (GIBCO) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin, 0·25 μg/ml amphotericin B, and immediately used for cytokine production or cryopreserved for flow cytometry or proliferation assays. Briefly, PBMC (1×10^7/ml) were suspended in 70% complete RPMI (as above), 20% fetal calf serum (FCS) and 10% DMSO and frozen at a cooling rate of 1°C per minute in liquid nitrogen.

Diagnosis of microfilaraemia

After Ficoll–Hypaque density gradient centrifugation, the red cell pellet and plasma was diluted in 20 ml of PBS (pH 7·2) and filtered through a polycarbonate filter (diameter 25 mm, pore size 5 μm, Nucleopore). Next, 10 ml of PBS and then air were passed through the same filter in order to wash off erythrocytes and to cause microfilariae to adhere to the filter. Subsequently, the filter was placed on a slide, covered with two drops of PBS, sealed with a cover slip, and examined under a microscope ($\times 400$) for the presence of microfilariae.

O. volvulus *antigen preparation*

Onchocerca nodules containing adult *O. volvulus* were excised and placed in sterile incubation containers together with RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphotericin B, 10% FCS and collagenase (5 mg/ml) (Boehringer), and incubated on a rocking plate at 32°C. Adult female and male worms were carefully isolated as described by Schulz-Key *et al.* [16]. The live and motile worms were washed in sterile PBS (pH 7·2–7·4) and snap frozen in liquid nitrogen. A PBS-soluble antigen extract was prepared under sterile conditions on ice in a Ten Broeck tissue grinder (Fisher), centrifuged at 4°C at 16000 *g* for 45 min and filtered through a 0·2 μm filter. The protein concentration of the extract as determined by the BCA method (Pierce) was 3·53 mg/ml. This antigen preparation was used throughout the study.

Cytokine assays

Cells were cultured at 5×10^6/ml in RPMI (as above) supplemented with 1% heat inactivated FCS, in the presence of either *O. volvulus*-derived antigen (OvAg) (3·5 μg/ml) or phytohaemagglutinin (PHA) (1:100, GIBCO) or streptolysin-O (1:50, Fisher) at 37°C and saturated humidity. Culture supernatants were collected after 24 or 48 h, filtered and stored in liquid nitrogen. IL-2 (Medgenix), IL-4 (Biermann) or IFN-γ (Holland Biotechnology) were quantified by ELISA or EASIA. The production of IL-1β, tumour necrosis factor-alpha (TNF-α) or IL-6 was measured by IRMA or EASIA (Medgenix). All assays were used as recommended by the manufacturer.

Proliferation assays

Cells were seeded at 1×10^5/well in sterile round-bottomed 96-well microtitre plates (Costar). They were suspended in RPMI (as above) containing 10% FCS, and were kept at 37°C in 5% CO_2 and saturated humidity. For mitogenic stimulation with PHA (1:100, GIBCO) and for antigenic stimulation with OvAg (3·5 μg/ml) or streptolysin-O (1:50, Fisher) cultures were maintained for 3 or 5 days respectively. For the last 18 h 1 μCi of ^{3}H-thymidine was added, cells were harvested on glass fibre filters (Skatron), and the incorporated radioactivity was determined by scintillation spectroscopy (Beta Plate, LKB-Pharmacia). Data were expressed as mean values of triplicate cultures in ct/min − ct/min of baseline stimulation without antigen or mitogen.

Flow cytometric analysis of lymphocyte populations

Two-colour flow cytometry was performed on PBMC by direct immunofluorescence staining. The following PE- or FITC-conjugated murine MoAbs specific for lymphocyte surface antigens were employed: anti-CD3 (pan T cell), anti-CD19 (B cell), anti-CD4 (T helper/inducer cell), anti-CD8 (T cytotoxic or suppressor cell), anti-HLA-DR (activation marker), anti-CD16 and anti-CD56 (natural killer cell) (Becton Dickinson), anti-CD23 (low-affinity receptor for IgE; Dianova), UCHL1-IgG2a-FITC (anti-CD45RO antigen on 'memory-cells'; Dianova) and anti-TCRδ1 (common epitope on delta chain of TCR; Biermann). Cryopreserved PBMC were thawed, resuspended and washed in RPMI. Cells (1×10^5) were incubated with MoAbs for 30 min at 4°C in dark. After two washes with sterile PBS (pH

Table 1. Delayed cutaneous hypersensitivity reactions to seven recall antigens in onchocerciasis patients before and after treatment with ivermectin, and of infection-free control individuals

	Patient group			Control group ($n=7$)
	Before therapy	6 months after therapy	14 months after therapy	
Males ($n=16$)				
Induration scores (mm)	9·4±6·2	16±5·8*	18·9±5·8†	15·5±5·8†
Positive reactions	2·3±1·5	3·6±1·5	4·7±1·3	4·3±1
Females ($n=5$)				
Induration scores (mm)	9·9±3·2	14·8±2·9	13·4±3·2	18·5±2·5†
Positive reactions	2·1±0·7	4·4±1·6	4±1	5·3±1

* Significantly different ($P<0·05$) compared with pretreatment values.
† Significantly different ($P<0·01$) compared with pretreatment values.

Table 2. Lymphocyte subpopulations of onchocerciasis patients before and after treatment with ivermectin, and of infection-free control individuals

	Patient group ($n=19$)					
Lymphocytes	Before therapy	1 week after therapy	1 month after therapy	6 months after therapy	14 months after therapy	Control group ($n=8$)
$CD3^+$	1279±613 (74%)	1619±509 (76%)	1840±635* (80%)	1859±665* (79%)	2063±748* (80%)	1788±662† (73%)
$CD19^+$	137±99 (8%)	187±110 (10%)	153±79 (7%)	155±99 (6%)	188±110 (7%)	215±77† (9%)
$CD23^+$	302±302 (16%)	230±156 (12%)	287±223 (13%)	279±281 (13%)	202±256 (7%)	232±111 (11%)
$CD4^+$	652±423 (38%)	909±418 (42%)	1026±369* (45%)	1002±469* (42%)	1093±410* (43%)	1162±470† (47%)
$CD8^+$	637±333 (38%)	671±247 (33%)	764±483 (32%)	832±366† (36%)	943±467† (36%)	594±154 (26%)
CD4/CD8	1·2	1·5	1·6*	1·3	1·4	1·9†
$TCR\delta1^+$ $CD3^+$	125±69 (10%)	95±43 (7%)	110±48 (7%)	128±70 (7%)	140±84 (7%)	65±23† (4%)
HLA-DR^+ $CD3^+$	268±187 (16%)	260±97 (13%)	393±201 (17%)	337±185 (14%)	376±285 (15%)	274±150 (11%)
$CD45R0^+$ $CD4^+$	403±193 (59%)	474±165 (52%)	602±222 (58%)	565±235 (57%)	730±232† (61%)	550±260 (49%)

Values are mean±s.d. of cells per microlitre. Mean values expressed in per cent CD^+ cells are in parentheses.
* Significantly different ($P<0·01$) compared with pretreatment values.
† Significantly different ($P<0·05$) compared with pretreatment values.

7·4) containing 0·1% NaN_3, cells were fixed in 3% formaldehyde and suspended in 500 µl of FACS sheath fluid. Cells were analysed with the FACScan System from Becton Dickinson. Data were collected from 1×10^4 cells per sample. Viability was determined by forward and side light scatter parameters. Background staining was determined by irrelevant mouse FITC- or PE-conjugated IgG1 and IgG2a. Background was less than 1%. Lymphocyte scatter gating was verified by Leucogate Simultest (anti-CD45-FITC and anti-CD14-PE), and the ratio of specifically stained cells to the total number of lymphocytes was calculated as a percentage. The absolute numbers of lymphocyte subpopulations were deduced from this percentage and from the total number of lymphocytes, as determined by the blood cell differential.

Delayed cutaneous hypersensitivity responses

Delayed cutaneous hypersensitivity (DCH) to seven ubiquitous recall antigens (tetanus and diphtheria toxoid-, Streptococcus-, tuberculin-, Candida-, Trichophyton- and Proteus-derived antigens) was determined with the commercially available Multitest Mérieux before ivermectin treatment and at 6 and 14 months after treatment. The antigens were applied intracutaneously on the forearm, as recommended by the manufacturer, and DCH reactions were quantified after 48 h by measurement of the induration scores in millimetres.

Statistical analysis

Statistical analyses were performed by either Student's *t*-test or the Mann–Whitney test. Analysis of DCH responses, of proliferative reactivity and of cytokine production was performed after logarithmic transformation of data.

RESULTS

Microfilaria of O. volvulus *and* Mansonella perstans

The mean initial density of *O. volvulus* microfilariae (mf) in 55 patients was 75·8 mf/mg skin and this dropped to 6·0 mf/mg and

Table 3. *In vitro* proliferative responses (ct/min $\times 10^{-3}$) of peripheral blood mononuclear cells (PBMC) to mitogenic and antigenic stimulation of ivermectin-treated patients, and of infection-free control individuals

Stimulation	Patient group ($n=19$)					Control group ($n=6$)
	Before therapy	1 week after therapy	1 month after therapy	6 months after therapy	14 months after therapy	
PHA	58·9±10·7	50·7±13·0	56·9±10·6	46·6±7·7	87·3±12·6	52·6±8·7
OvAg	2·8±1·0	0·8±0·3	2·2±0·8	2·6±1·3	5·5±1·5	8·8±1·7*
SL-O	49·2±17·1	37·6±12·2	44·2±11·3	53·7±12·0	95·8±9·2*	90·6±11·9

* Significantly different ($P<0·05$) compared with pretreatment values.

Results are expressed as mean ct/min ± s.e.m.; i.e. ct/min with stimulation − medium control without mitogen or antigen.

Phytohaemagglutinin (PHA), 1:100; *O. volvulus*-derived antigen (OvAg), 3·5 μg/ml; streptolysin (SL-O), 1:50.

Table 4. Production of cytokines by peripheral blood mononuclear cells (PBMC) in response to mitogen and *O. volvulus* antigen in patients before and after ivermectin treatment, and in infection-free control individuals

Cytokines and stimulation	Patient group			Control group ($n=6$)
	Before therapy	1 month after therapy	14 months after therapy	
IL-1β (pg/ml) ($n=11$)				
PHA	2254±900	2149±936	2497±692	ND
OvAg	0±0	551±364*	1598±433†	ND
TNF-α (pg/ml) ($n=12$)				
PHA	4678±1239‡	5205±971	4583±1127	ND
OvAg	0±0	83±63*	542±249†	ND
IL-6 (pg/ml) ($n=14$)				
PHA	236±119	218±117	693±280	425±237
OvAg	1388±433	948±431	679±273	475±287
IL-2 (U/ml) ($n=7$)				
PHA	0·36±0·1	9·3±3*	3·3±1*	4·9±3·5*
OvAg	0·7±0·3	0·6±0·3	0·2±0·1	0·3±0·2
IL-4 (pg/ml) ($n=24$)				
PHA	199±76	623±113*	506±87*	270±123
OvAg	13±7	12±5	24±12	1·8±1·6*
IFN-γ (U/ml) ($n=24$)				
PHA	45±9	49±9	50±9	76±14
OvAg	19±5	13±7	30±10	0·25±0·3*
IL-4/IFN				
PHA	4·4	12·7	10·1	3·5
OvAg	0·7	0·9	0·8	7·2

* Significantly different ($P<0·05$) compared with pretreatment values.

† Significantly different ($P<0·01$) compared with pretreatment values.

‡One week after treatment.

Phytohaemagglutinin (PHA), 1:100; *O. volvulus*-derived antigen (OvAg), 3·5 μg/ml.

ND, Not determined.

13·3 mf/mg at 6 and 14 months after treatment respectively. Initially, 67% of all patients were positive for blood-dwelling microfilariae of *M. perstans*. After therapy mansonelliasis was diagnosed in 62%, 73% and 36% of the patients at 1, 6 and 14 months after treatment.

DCH reactions

The DCH responses were significantly lower in untreated patients than in infection-free controls; moreover, 7/16 males were hyporeactive (scores <10 mm) (Table 1). Skin test reactivity to seven recall antigens increased after therapy in both

males and females, but remained unchanged in patients not treated with ivermectin. At 6 months after treatment in both males and females the number of positive reactions and the induration scores had approached values found in male and female control individuals.

Total leucocyte counts, eosinophilia and lymphocyte subsets before and after treatment

Mean leucocyte counts of infection-free control individuals and of untreated onchocerciasis patients were within the normal range. At 1 month after treatment total leucocytes and granulocytes transiently increased before returning to similar values as seen before initiation of therapy. Mean eosinophil counts dropped significantly until 14 months after treatment, but always remained higher in patients than in local controls. Before therapy one-third of all patients were lymphocytopenic ($< 1{\cdot}200/\mu l$), but lymphocyte counts normalized after therapy and at 14 months after treatment approached the same values ($2403/\mu l$, 44%) as found in the infection-free control group ($2430/\mu l$; 44%).

Before therapy, circulating mature T cells ($CD3^+$) of patients were reduced compared with infection-free controls (Table 2). The relative and absolute number of $CD3^+$ cells increased after therapy, remaining significantly augmented from 1 month after treatment onwards so that at 14 months after treatment $CD3^+$ cells/μl in patients were approximately the same as in control individuals. In parallel, T helper cells ($CD4^+$) increased continuously after therapy, becoming statistically higher by 1 month after treatment ($P < 0{\cdot}01$); at 14 months after treatment the $CD4^+$ counts had approached control values. Throughout the period of observation more $CD8^+$ cells were found in patients than in controls. Absolute numbers of $CD8^+$ cells increased progressively following ivermectin treatment, exceeding pretreatment levels at 6 months and 14 months after treatment. The initial impaired $CD4^+/CD8^+$ ratio transiently improved at 1 month after treatment ($P < 0{\cdot}01$), but decreased thereafter. The patients' $CD4^+/CD8^+$ ratio was always lower than in controls. Fewer resting $CD19^+$ B cells were found in untreated patients than in controls and the number of $CD19^+$ B cells did not change following therapy. The low-affinity receptor for IgE (CD23) was expressed on 16% of total lymphocytes of untreated patients and $CD23^+$ cells decreased to 7% 14 months after treatment. The $\gamma\delta$ T cell receptor (TCR) was found on 10% ($125/\mu l$) of $CD3^+$ cells of patients, which was higher than in controls. Following therapy the numbers of TCR $\gamma\delta^+$ cells remained unchanged. Before therapy, T cells which expressed $HLA\text{-}DR^+$ were present in similar numbers in both patients and controls. One month after treatment, the number of $CD3^+HLA\text{-}DR^+$ cells peaked to 17% ($393/\mu l$), remaining above pretreatment values at 14 months after treatment. The $CD45RO^+$ cells (memory cells) initially constituted 59% ($403/\mu l$) of $CD4^+$ cells in patients. The absolute numbers of $CD4^+CD45RO^+$ cells increased after therapy, and with 61% ($730/\mu l$), at 14 months after treatment exceeded values found in controls (49%, $550/\mu l$).

Cellular responses to mitogen and antigen

In the range investigated, *in vitro* cellular responses of PBMC to PHA were similar in both patients and controls (Table 3). PBMC from patients proliferated vigorously to streptolysin O (SL-O); however, proliferation was clearly less than that seen with PBMC from controls. At 14 months after treatment, the mean cellular reactivity to SL-O in patients ($n = 19$) had approached the responses found in controls ($n = 6$). Before therapy, proliferative responses to OvAg were lower in patients (2815 ct/min) than in controls (8836 ct/min) ($P < 0{\cdot}05$). One week after ivermectin, the mean responses to OvAg had decreased to 832 ct/min, but thereafter cellular reactivity increased continuously to 2188, 2640 and 5508 ct/min at 1 month, 6 months and 14 months after treatment respectively.

Cytokine production

PBMC from untreated onchocerciasis patients and from control individuals produced significant amounts of IL-1β, IL-4, IL-6, IFN-γ and TNF-α in response to mitogenic stimulation with PHA, but only low levels of IL-2 (Table 4). However, PHA-driven IL-2 production by PBMC from infection-free controls was higher than that of untreated patients, whereas mitogen-induced IL-4 was similarly high in both groups. OvAg-driven secretion of IL-4 and IFN-γ by PBMC was higher in onchocerciasis patients than in infection-free controls. After ivermectin therapy, OvAg-induced secretion of IL-1β and TNF-α increased significantly at 1 month after treatment, and remained elevated until 14 months after treatment. Simultaneously, PHA-induced production of IL-2 and IL-4 was augmented at 1 and 14 months after treatment. OvAg-specific secretion of IL-2, IL-4 and IFN-γ did not change after ivermectin treatment. The ratio of IL-4 and IFN-γ production in response to mitogenic stimulation indicated preferential IL-4 production, which increased further after ivermectin treatment.

DISCUSSION

Infection with *O. volvulus* is associated with an impaired cell-mediated immune response of the host [2,3]. Sequential *in vivo* testing of DCH is a suitable method for assessing even subtle alterations in cellular immunity, with failure to respond to a battery of recall antigens indicating cutaneous anergy or hypoergy [17]. In our study, onchocerciasis patients showed such diminished skin test reactivity; moreover, this accords with previous observations [18,19]. As a result of ivermectin therapy DCH reactions in both males and females normalized, indicating a clear improvement in cellular immune functions after reduction of *O. volvulus* microfilariae. Since patients in areas endemic for onchocerciasis suffer from multiple parasitic infections and parasite-induced cellular anergy may increase susceptibility to concurrent infections, observed normalization of DCH reactions in our patients was an important outcome of ivermectin treatment.

The dramatic reduction of *O. volvulus* microfilariae after therapy was followed by a gradual increase in the number of circulating $CD4^+$ T cells, and an augmented cytokine production by PBMC. The number of $CD4^+$ T cells doubled within 1 month after treatment, and by 14 months after treatment had reached the same levels as found in controls. Immune responses to recall antigens are initiated preferentially by *in vivo* primed (memory) $CD4^+CD45RO^+$ T cells [20], which have acquired the ability to synthesize both IL-2 and IL-4 mRNA [21]. In the onchocerciasis patients studied, 59% of $CD4^+$ T cells were $CD45RO^+$, while 49% of such cells were found in control individuals. Moreover, by 14 months after treatment, the numbers of patients' $CD4^+CD45RO^+$ cells exceeded those

found in controls. Following ivermectin treatment, microfilariae of *O. volvulus* are drained to the local lymph nodes and degraded [22]. As a sequel of these events, accessory cells and lymphocytes may have encountered *de novo* microfilarial antigens, lymphocytes recirculated, appearing as $CD4^+CD45RO^+$ in the peripheral blood and possibly migrating into the skin.

The inability of PBMC to respond to specific parasite antigens may depend on the number of $CD4^+$ cells and on parasite-specific cytokine secretion, both of which were critically reduced in untreated patients. *In vitro* cellular reactivity rose gradually and peaked at 14 months after ivermectin treatment; such responsiveness may possibly have been supported by the synergistic action of several cytokines. IL-1β and TNF-α are major products of stimulated monocytes and lymphocytes; both induce *in vitro* increased synthesis of IL-2, IL-4, IL-6 and colony stimulating factors [23]. As previously shown, cells from onchocerciasis patients generate minimal amounts of IL-1 in response to OvAg; however, significant levels of IL-1 are generated when stimulated with lipopolysaccharide (LPS) [3]. Increased production of IL-1β and TNF-α, induced after ivermectin treatment, may have activated accessory cells and T lymphocytes which then, via up-regulation of IL-2 and IL-4, facilitated specific cellular reactivity against *O. volvulus*. Indeed, both IL-2 and IL-4 secretion by mitogen-stimulated PBMC was increased after therapy. Deficient production of IL-2, as observed in human and experimental filariases [3,6,24], is probably not the sole defect of cellular immunity. *In vitro* cellular responses were partially augmented by exogenous IL-2 in onchocerciasis patients [3], but IL-2 was unable to reconstitute suppressed parasite-specific cellular responses in experimental filariases [6,24]. IL-4 stimulates proliferation of preactivated T cells independently of IL-2, and together IL-2 and IL-4 support a greater degree of proliferation than each cytokine alone [25]. Also, a defective production of IFN-γ is thought to contribute to the multifactorial deficiency in cellular immunity in filariases [1]. These observations indicate that multifactorial events are necessary to reverse the depressed cellular immune response of filariasis patients, and cytokines, acting in synergy, may have to reach a threshold level for ultimate T cell activation.

Even though this study supports the strong influence of cytokines on cellular responsiveness, we cannot clearly differentiate Th1 and Th2 type responses [26,27] in onchocerciasis patients. The ratio of IL-4 to IFN-γ increased after therapy in response to mitogenic stimulation, possibly indicating preferential activation of Th2 type responses. In onchocerciasis patients, T cell activation seems to depend on the complex cytokine network and may be restricted not only to Th1 or Th2 $CD4^+$ phenotypes, but may also include $CD8^+$ cells. However, the functional importance of $CD8^+$ cells in metazoic (extracellular) parasitic infections remains to be defined more precisely. In brugian filariasis patients, $CD8^+$ cells were found to be markedly elevated and were identified as antigen-specific suppressors of immune responses [28]. In the present study, absolute numbers of $CD8^+$ cells were higher in patients than in uninfected controls, and further increased following therapy. For the human $CD8^+$ cell population a functional dichotomy into two subsets was suggested: namely, $CD8^+$ cytolytic cells (CTL) that secrete IFN-γ and IL-10; and Type 2 $CD8^+$ T-suppressor cells (Ts) that produce IL-4 [29]. Increased IL-4 production, facilitated by ivermectin therapy, might have activated $CD8^+$ Ts cells and thus counteracted cellular reactivity to OvAg in patients. However, this aspect needs validation.

In this study, untreated patients had fewer resting B cells ($CD19^+$) than controls, but a higher expression of the low-affinity receptor for IgE (FcεRII/CD23). Only half as many $CD23^+$ cells were present at 14 months after treatment. The CD23 molecule is involved in B cell growth and differentiation, regulates IgE synthesis by B cells and participates in the killing of schistosomulae by eosinophils [30]. Recently, it was shown by Yamaoka *et al.* [31] that excretory and secretory [ES] antigens of *Dirofilaria immitis* induce the expression of CD23 on human T cells. The decreasing number of $CD23^+$ cells in treated patients indicates minor activated B cells, suggesting diminishing production of OvAg-specific IgE, low levels of circulating ES-antigens of *O. volvulus* and, possibly, a down-regulated cellular cytotoxic activity against microfilariae.

A small subpopulation of $CD3^+$ T cells express the $\gamma\delta$ TCR, these cells frequently being found in epidermal tissues [32], and $TCR\gamma\delta^+$ cells are of functional importance in several infectious diseases and immune deficiencies [33]. In our study, more TCR $\gamma\delta^+$ cells were found in the peripheral blood of patients than in controls, and numbers remained augmented following therapy. The possible contribution of $CD8^+$ or TCR $\gamma\delta^+$ cells to immunosurveillance in onchocerciasis, particularly in the skin, remains to be examined further.

Two-thirds of our onchocerciasis patients were concomitantly infected with *M. perstans*, but the extent to which *M. perstans* induces pathology or immunosuppression remains unknown. Recently, Albrecht *et al.* [34] have shown that ivermectin only partially reduces the microfilariae of *M. perstans* to an average of 68% of the pretreatment level; it remains unclear whether *M. perstans* infection interferes with the induced restoration of immune response after ivermectin.

The results presented in this study suggest a distinct alteration of immunity in ivermectin-treated humans chronically infected with *O. volvulus*. The augmentation of DCH reactions, the normalization of several lymphocyte subpopulations, and the increased production of cytokines may have supported or enhanced parasite clearance. Permanent clearance of microfilariae cannot be achieved, and the weak efficacy of ivermectin on adult *O. volvulus* and on concomitant *M. perstans* may restrict drug-facilitated immunity. However, the most striking and exciting finding of ivermectin treatment remains, that a single and extremely low dose is able to induce substantial and long-lasting changes of cellular immunity in onchocerciasis patients.

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